# Caffeine Inhibition of Aflatoxin Production: Mode of Action

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Evaluation of caffeine and a number of related methylxanthines indicated that the ability of the compound to inhibit growth and aflatoxin production by *Aspergillus parasiticus* is highly specific and does not involve an inhibition of cyclic AMP phosphodiesterase. Supplementation of the culture medium with purine bases, nucleosides, and nucleotides suggested that the inhibition of fungal growth could be partially overcome by adenine or guanine but that the purines had little effect on the inhibition of aflatoxin production. Likewise, increasing the levels of trace minerals did not overcome the inhibition of toxin production. Electron microscopic evaluation of caffeine-treated and -untreated cultures indicated that the compound produced observable changes in the ultrastructure of the fungus.

Caffeine (1,3,7-trimethylxanthine) has been shown to inhibit growth and polyketide mycotoxin production in a number of *Aspergillus* and *Penicillium* species (6, 6a, 7), but little is known about its mode of action. The inhibitory activity of caffeine appears to be highly specific, since closely related dimethylxanthines (theophylline and theobromine) had little effect on mycotoxin formation (5–7). Potentially, the identification of the caffeine inhibition mechanism of polyketide synthesis could be useful for better understanding the bioregulatory processes controlling mycotoxin formation. The objective of the present study was to further characterize the activity of caffeine by assessing a number of possible modes of action, using aflatoxin production by *Aspergillus parasiticus* as a test system.

## MATERIALS AND METHODS

**Microorganisms.** *A. parasiticus* NRRL 2999 was used throughout the study. Stock cultures were maintained on potato glucose agar (Difco Laboratories) slants stored at 4°C. Spore suspensions were prepared as previously described (22).

**Medium.** YES medium (2% yeast extract, 6% sucrose) (9) was used throughout the study.

For the trace metal supplementation study, a stock solution of the metal mix described by Mateles and Adye (15) was prepared by dissolving $Na_2B_4O_7 \cdot 10H_2O$ (700 mg), $NaMoO_4 \cdot 2H_2O$ (680 mg), $FeSO_4 \cdot 7H_2O$ (10 g), $CuSO_4 \cdot 5H_2O$ (300 mg), $ZnSO_4 \cdot 7H_2O$ (1.76 g), and $MnSO_4 \cdot H_2O$ (110 mg) in 1,000 ml of distilled, deionized water. YES medium with $MgSO_4$ (2 g/liter) and containing 0 and 2 mg of caffeine per ml was then supplemented with the metal mix at levels of 0, 50, and 500 µl/50 ml.

**Culture techniques.** YES medium containing 0 or 2 mg of caffeine per ml was transferred in 50-ml portions to 250-ml Erlenmeyer flasks, capped, and autoclaved for 10 min at 15 lb/in². Other supplements added to the medium were introduced before or after autoclaving, depending on their heat stability. All flasks were inoculated with 0.1 ml of spore suspension to produce an inoculum of approximately $2 \times 10^4$ conidia per ml. Unless otherwise specified, all flasks were incubated without agitation for 7 days at 28°C. The cultures were then analyzed for growth and aflatoxin production. At least three cultures were employed for each variable combination and compared against appropriate controls.

Cultures employed for electron microscopic studies were grown in a similar manner; however, to facilitate the preparation of sections, the cultures were incubated on a rotary shaker (120 rpm), and samples were removed after 3 and 5 days.

**Aflatoxin analysis.** Aflatoxins (mycelium plus medium) were extracted and concentrated as previously described (22) and subsequently cleaned by the silica gel cartridge technique of McKinney (16). (Aflatoxins are highly toxic and carcinogenic and should be handled with extreme care.) Aflatoxins were separated by reversed-phase, high-pressure liquid chromatography (model 410, Altrex, Inc.) with a linear gradient of 0.04% formic acid in water to methanol (25- by 4.5-cm column; flow rate, 1.0 ml/min, $C_{18}$, 5 µm; Altrex). Detection was achieved by monitoring the absorption at 360 nm, and quantitation was by comparison of the peak heights against those of known standards.

**Dry weight determinations.** After aflatoxin extraction, the mycelia were transferred to preweighed filter paper (Whatman no. 1), rinsed with distilled water, and dried for 18 h at 85°C. The mycelia were then cooled in a desiccator, and the mycelial dry weights were determined gravimetrically.

**Phosphodiesterase determination.** Enzyme extracts were prepared from 3-, 6-, 8-, and 10-day mycelia by using a modification of the procedure described by

Niehaus and Dilts (17). Mycelium was harvested on cheesecloth and immediately frozen in a liquid nitrogen-hexane mixture. The samples were lyophilized and subsequently extracted twice with acetone (ca. 30 ml/g). Residual solvent was removed under vacuum, and the samples were stored in a desiccator at −80°C. Samples were rehydrated by adding 3.0 ml of cold buffer (50 mM potassium phosphate [pH 7.2], 1 mM EDTA, and 2 mM mercaptoethanol, with 0.5 mM phenylmethylsulfonyl fluoride [Sigma Chemical Co.] added just before use) per 100 mg of mycelium. The cells were disrupted with a glass mortar and pestle held on ice, and the resulting suspension was centrifuged at 7,000 × $g$ for 10 min at 4°C. The supernatant was employed as the crude enzyme extract and was used immediately. The protein contents of the enzyme extracts were determined by the technique of Lowry et al. (12).

The effect of caffeine and theophylline on cyclic AMP (cAMP) phosphodiesterase activity was determined in 1.5-ml, capped Eppendorf vials. A 0.1-ml portion of enzyme extract was mixed with 0.1 ml of inhibitor solution and incubated for 1 h at 30°C. The cAMP solution (0.1 ml) was added, rapidly mixed, and incubated for 15 min. The reaction was then stopped by addition of 0.5 ml of cold 400 mM $HClO_4$. The contents were centrifuged, and the supernatant was saved for subsequent analysis. Caffeine (0, 30, and 90 mM) and theophylline (0, 10, 30, and 90 mM) were tested in conjunction with cAMP concentrations of 10, 15, and 20 mM.

Phosphodiesterase activity was monitored by determining residual cAMP concentrations by using a modification of the high-pressure liquid chromatographic technique of Kristulovic et al. (11). A reversed-phase column ($C_{18}$, 5 μm, 25 by 4.6 cm; Altrex) was employed in conjunction with a mobile phase consisting of a linear gradient of 0 to 98% methanol in a 0.04% formic acid solution. A flow rate of 1.0 ml/min was maintained, and cAMP was detected by monitoring the absorption at 254 nm. Quantitation was achieved by comparison of sample peak heights against those of known standards.

**Electron microscopy.** Mycelial pellets from agitated cultures containing 0 and 2 mg of caffeine per ml were fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for 4 h at room temperature and postfixed in 2% osmium tetroxide in Palade buffer (18) for 4 h. Samples were dehydrated with an ethanol series followed by propylene oxide and were embedded in Epon epoxy resin. Thin sections stained with uranyl acetate and lead citrate were observed with a Zeiss electron microscope (model EM 10B) at 50 kV.

## RESULTS AND DISCUSSION

The specificity of caffeine as an inhibitor of aflatoxin synthesis was further characterized by determining the relative activity of a number of related compounds against *A. parasiticus* cultured in YES medium for 7 days. These results are summarized in Table 1 in terms of the percentage of untreated controls.

Caffeine was strongly inhibitory, with aflatoxin production being affected more than growth. These results are similar to those previ-

TABLE 1. Growth and aflatoxin production by *A. parasiticus* in medium containing various methylxanthines and related compounds[a]

| Supplement[b] | Mycelium (mg)[c] | Aflatoxin (μg/culture) | Aflatoxin/ mycelium (μg/mg) |
|---|---|---|---|
| None | 777 (100.0) | 2,313 (100.0) | 2.96 (100.0) |
| Caffeine | 65 (8.4) | 2 (<0.1) | 0.02 (0.7) |
| Theophylline | 609 (78.4) | 2,063 (89.2) | 3.39 (114.3) |
| Theobromine | 644 (82.9) | 2,098 (90.7) | 3.26 (109.9) |
| Isobutylmethylxanthine | 447 (57.5) | 1,029 (44.5) | 2.30 (77.7) |
| Xanthine | 777 (100.0) | 1,269 (54.9) | 1.63 (55.1) |
| Hypoxanthine | 581 (74.8) | 1,608 (69.5) | 2.77 (93.4) |

[a] Mean of triplicate cultures. Value in parentheses is the average percent response as compared with unsupplemented control cultures.
[b] All compounds added at a concentration of 2 mg/ml.
[c] Dry weight.

ously reported for aflatoxins (6) and other polyketide mycotoxins (6a, 7). Likewise, the lack of activity for theophylline and theobromine is similar to the results of previous investigations (5, 6, 7). Isobutylmethylxanthine and hypoxanthine decreased growth to some extent but had less effect on aflatoxin production. Conversely, xanthine did not affect growth, but it did depress mycotoxin production by approximately 45%. Strict interpretation of the data is difficult since the maximum solubilities of theobromine, isobutylmethylxanthine, xanthine, and hypoxanthine were exceeded by the concentration of 2 mg/ml that was employed. Attempts to find an acceptable solubilizing agent were unsuccessful. The results confirm the specificity of caffeine as an inhibitor of mycotoxin synthesis, which suggests that its mode of action may involve a highly specific binding site. The data further suggest that the mode of action of caffeine does not involve an inhibition of cAMP phosphodiesterase. Buchanan and Fletcher (6) hypothesized that the inhibition of aflatoxin production by caffeine could involve an inhibition of cyclic nucleotide phosphodiesterase. However, Tice and Buchanan (22) found that exogeneously supplied cAMP stimulated aflatoxin synthesis and suggested that the mode of action of caffeine did not involve altered phosphodiesterase activity. Theophylline and isobutylmethylxanthine are generally considered to be at least as effective as caffeine as inhibitors of cAMP phosphodiesterase (8, 10). Their lack of anti-aflatoxigenic activity in the present study further suggests that the mode of action of caffeine does
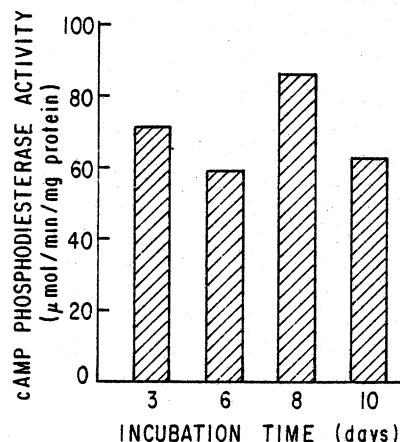
FIG. 1. cAMP phosphodiesterase activity of *A. parasiticus* cultured in YES medium for 3, 6, 8, and 10 days.

not involve an inhibition of phosphodiesterase activity.

To better assess the role of cAMP phosphodiesterase as a possible site of action for caffeine, the in vitro inhibitory activity of caffeine and theophylline against this enzyme were compared. The cAMP phosphodiesterase activity of 3-, 6-, 8-, and 10-day mycelia is depicted in Fig. 1. Small fluctuations in cAMP phosphodiesterase activity with culture age were observed, but overall, enzyme activity remained relatively constant. Fluctuations in phosphodiesterase activity during growth and differentiation have been reported in other fungal species such as *Mucor rouxii* (19) and *Blastocladiella emersonii* (14). The 3-day mycelia were used to determine the inhibitory activity of caffeine and theophylline.

Lineweaver-Burke plots of the effects of caffeine and theophylline on cAMP phosphodiesterase activity are depicted in Fig. 2. Both methylxanthines inhibited the enzyme, with theophylline being approximately 20% more inhibitory than caffeine at comparable concentrations. This is in general agreement with other investigations (8, 10) that have indicated that in eucaryotic systems, theophylline is a more effective cAMP phosphodiesterase inhibitor than caffeine. Assuming that there is no limitation of uptake, these data strongly suggest that the mode of action of caffeine as an inhibitor of polyketide mycotoxin synthesis does not involve an inhibition of cAMP phosphodiesterase.

Caffeine has been shown to have a number of other biological effects. One such effect appears to involve a disruption of the normal biochemical functioning of purines or their corresponding nucleosides or nucleotides. For example, Snyder et al. (21) hypothesized that the caffeine-

mediated stimulation of mammalian behavior is due to the ability of the molecule to competitively block neurological adenosine receptors. Bard et al. (4) found that caffeine inhibited the growth of both petite and grande strains of *Saccharomyces cerevisiae*. They further reported that the addition of adenine partially overcame the inhibition of the grande strain, but had no effect on the petite strain. To determine whether the inhibition of growth and toxin production in *A. parasiticus* by caffeine may involve a disruption of purine metabolism, cultures were supplemented with adenine, guanine, or their corresponding nucleosides and nucleotides (Table 2).

None of the compounds tested greatly relieved the inhibition of aflatoxin production by caffeine. Supplementation with adenine, adenosine, or guanine partially overcame the inhibition of growth by caffeine. A slight increase in aflatoxin production was observed in these cultures; however, this was small in comparison with the growth effects. These results suggest that the inhibition of growth, but not polyketide synthesis, may in part be due to a disruption of purine-associated processes. This is in agreement with Buchanan et al. (6a), who suggested that caffeine inhibition of growth and mycotoxin formation are not directly related. The ability of adenine to partially overcome growth inhibition
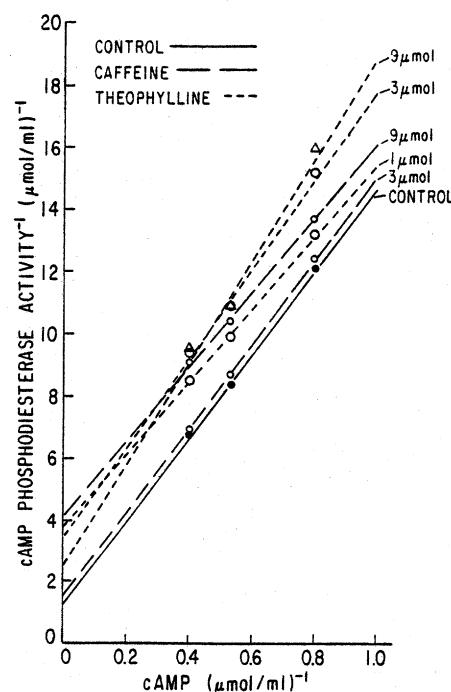


FIG. 2. In vitro inhibitory activity of caffeine and theophylline against cAMP phosphodiesterase from *A. parasiticus* cultured for 3 days in YES medium.
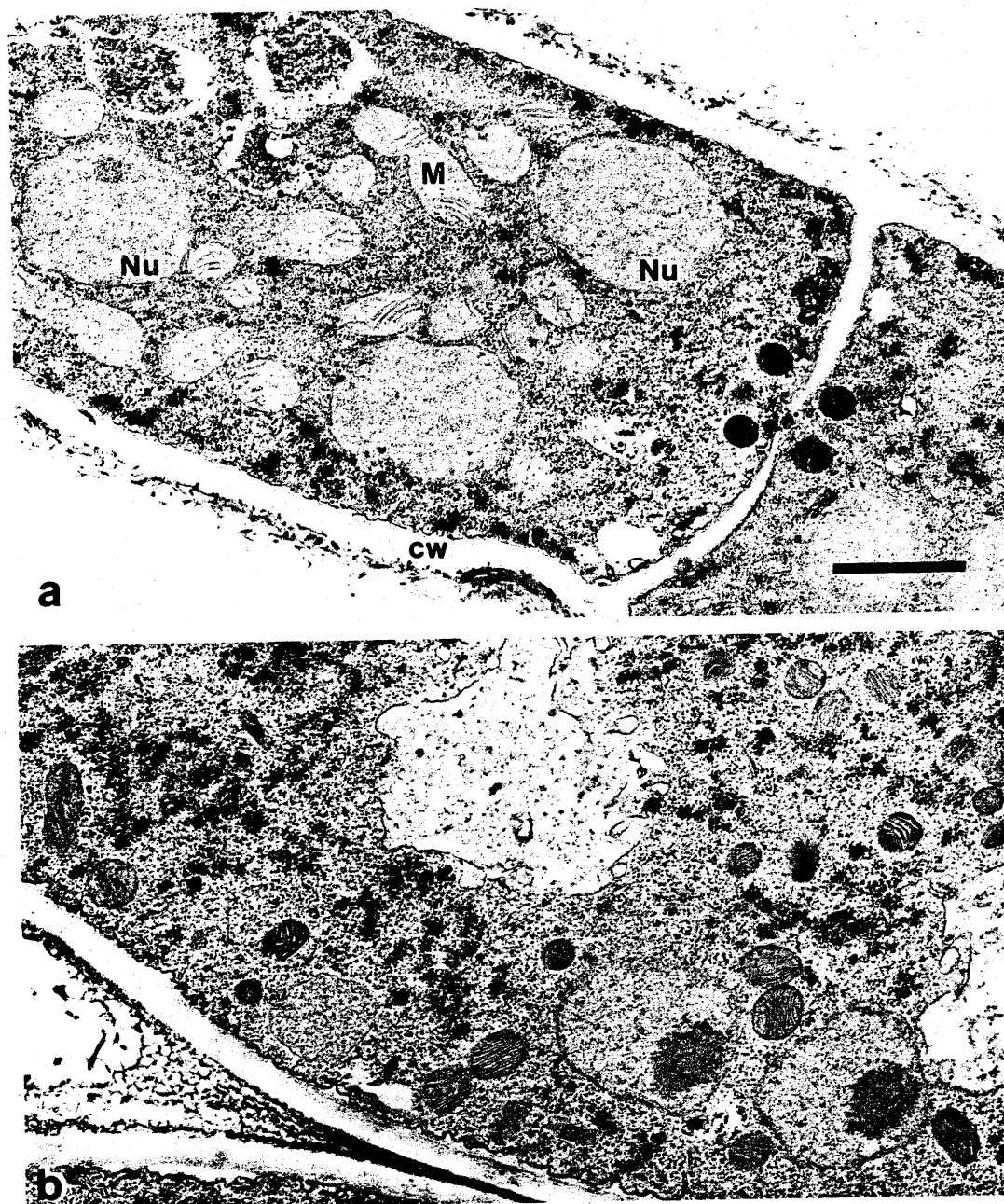
FIG. 3. Electron micrographs (×18,500) of agitated cultures of *A. parasiticus* NRRL 2999 grown for 3 and 5 days in YES medium containing 0 and 2 mg of caffeine per ml. (a) 3 day, 0 mg/ml; (b) 5 day, 0 mg/ml; (c) 3 day, 2 mg/ml; (d) 5 day, 2 mg/ml. CW, Cell wall; Nu, nucleus; M, mitochondrion; V, vacuole. Bar = 1.0 µm.

by caffeine is similar to the results reported by Bard et al. (4) for *S. cerevisiae*. However, in that study both caffeine and theophylline inhibited growth, and their effects could not be overcome by the addition of guanine.

In the absence of caffeine, a number of compounds (adenine, AMP, cAMP, GMP, cyclic GMP) inhibited toxin production without greatly affecting growth. Inhibition of toxin synthesis by GMP has been observed previously (22). The stimulation of aflatoxin production by cAMP supplementation previously reported by Tice and Buchanan (22) was not detected in the present study. However, this was not unexpected since the present study employed YES medium, whereas the earlier experimentation used a
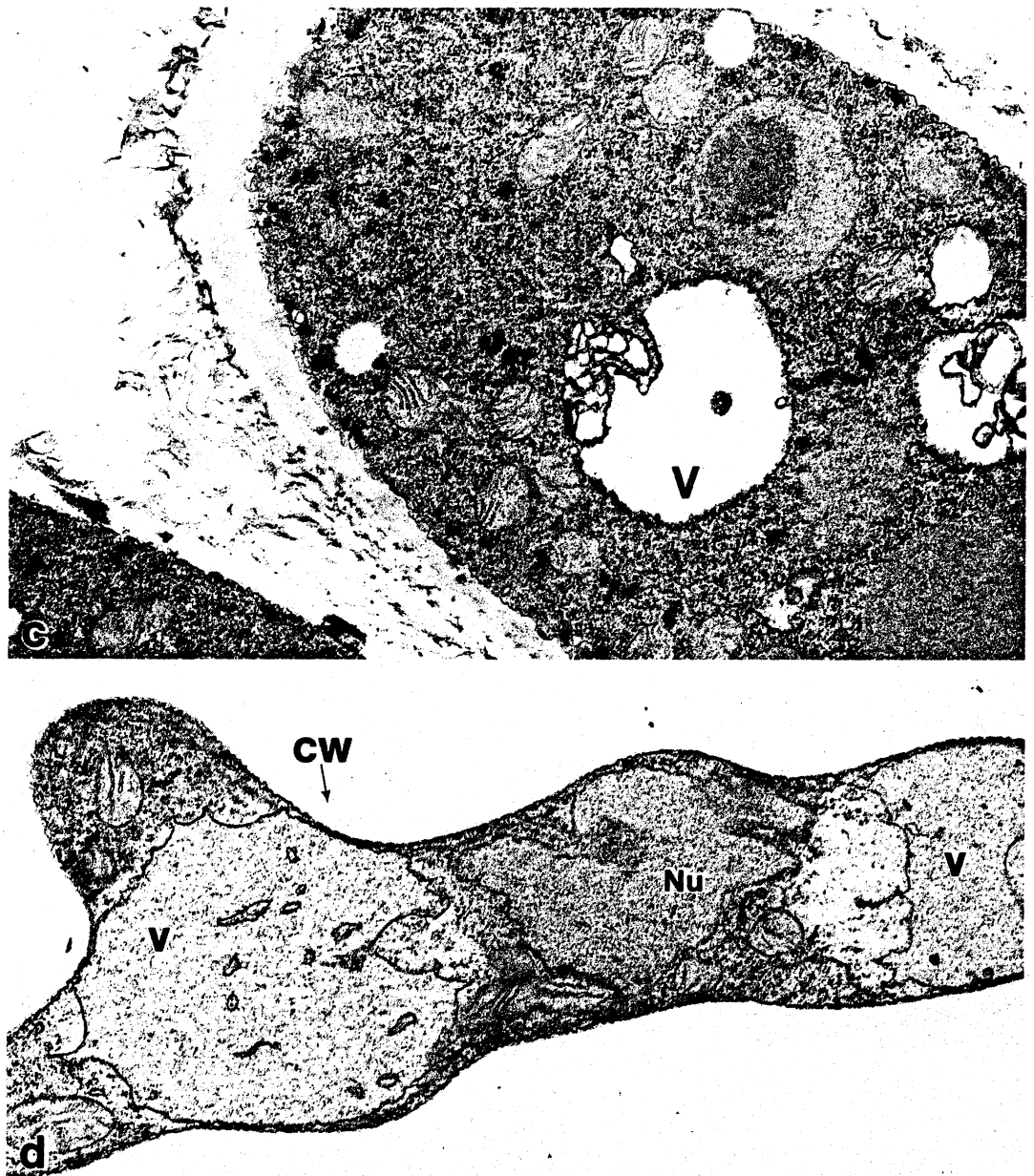
FIG. 3—*Continued*

glucose-mineral salts medium. Preliminary studies in our laboratory (unpublished data) indicate that the addition of yeast extract to glucose-mineral salts medium eliminates or masks the stimulatory effect of cAMP.

Aflatoxin synthesis is influenced greatly by the availability of metal ions (13). Among the various biological effects attributed to the methylxanthines, compounds of this class have been reported to complex with metal ions. Specifical-

ly, Tu and Friederich (24) reported that theophylline, but not caffeine, was active. This suggests that the inhibition of aflatoxin biosynthesis by caffeine does not involve an alteration in the bioavailability of key trace metals. To verify this supposition, the inhibitory activity of caffeine was examined in YES medium supplemented with $MgSO_4$ and two levels of the trace mineral mixture of Mateles and Adye (15) (Table 3). As anticipated, the increased trace mineral content

TABLE 3. Effect of increased levels of trace minerals on caffeine inhibition of growth and aflatoxin production by *Aspergillus parasiticus*[a]

| Trace metal supplement[b] | Caffeine[c] | Mycelium (mg)[d] | Aflatoxin (μg/culture) | Aflatoxin/mycelium (μg/mg) |
|---|---|---|---|---|
| 0 | − | 1,103 (100.0) | 2,596 (100.0) | 2.35 (100.0) |
| | + | 76 (6.9) | 0 (0.0) | 0.00 (0.0) |
| 50 | − | 1,243 (112.7) | 3,354 (129.2) | 2.69 (114.6) |
| | + | 45 (4.1) | 0 (0.0) | 0.00 (0.0) |
| 500 | − | 1,259 (114.1) | 2,602 (100.2) | 2.07 (87.8) |
| | + | 114 (10.3) | <1 (<0.1) | <0.01 (0.1) |

[a] Mean of triplicate cultures. Value in parentheses is the average percent response as compared with unsupplemented control cultures.
[b] Microliters of trace mineral stock solution added to 50 ml of medium.
[c] 2 mg/ml.
[d] Dry weight.

treated cells were greatly reduced in size. Hyphal diameter in treated cells was 30 to 50% that of the controls. The ultrastructure, and light microscopy of methylene blue-stained epoxy sections, showed large, filled vacuoles alternating with cytoplasmic regions along the hyphae. Nuclei in these cells were notably irregular in shape, and mitochondria often appeared to be elongated. In addition, the fibrous cell wall covering was absent on 5-day caffeine-treated cells. During sample handling, it was found that caffeine-treated mycelia were more friable than the controls. It is possible that the observed extracellular material plays a role in "glueing" the mycelium together.

The present study examined several possible modes of action to determine whether they could account for the ability of caffeine to inhibit growth and mycotoxin production by fungi. The results indicate that the growth inhibition was, in part, due to an alteration of either purine metabolism or purine function. However, this did not appear to account for the inhibition of aflatoxin production. Likewise, inhibition of toxin synthesis does not involve an inhibition of cAMP phosphodiesterase or a chelation of key metal ions.

Caffeine has been reported to have multiple effects in various systems. In addition to inhibiting cAMP phosphodiesterase and blocking nucleoside receptors, caffeine has been reported to alter DNA polymerase I (3) and RNA polymerase I (20) activity. Recently, Tortora et al. (23) reported that caffeine, but not theophylline or papaverine, uncoupled the regulation of glycolysis and gluconeogenesis in *S. cerevisiae*. Specifically, caffeine appeared to inhibit the carbon catabolite inactivation of fructose-1,6-diphosphatase, resulting in depressed glycolytic activity and altered energy charge. Aflatoxin synthesis has been reported to be highly dependent on the active catabolism of suitable carbohydrates (1, 2), and reduced glycolytic activity would be expected to strongly depress polyketide synthe-

sis. Work is currently under way to determine whether the inhibition of polyketide mycotoxin synthesis by caffeine involves an alteration in the regulation of glycolysis and gluconeogenesis.

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